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(54) Title: METALLOTHIONEIN BASED NEURONAL THERAPEUTIC AND THERAPEUTIC METHODS

(57) Abstract: A method of stimulating neuronal growth or repair comprising exposing a target neuron or neuronal area to a solution of the metallothionein isoform MT-IIA.

Metallothionein Based Neuronal Therapeutic and Therapeutic Methods

Introduction to the Invention

This invention relates to the use of metallothionein as an active ingredient in effecting and enhancing recovery of damaged neuronal tissue, particularly following physical trauma and damage thereto. The invention provides a therapeutic incorporating metallothionein and methods of treatment based thereon.

Background to the Invention

Metallothionein (MT) is a naturally occurring peptide, which is present in most cells of the mammalian body. There are many isoforms in humans, but these resolve into four classes; MT-I and MT-II which are expressed widely, MT-III which is mainly found in the brain, and MT-IV which is restricted to specific epithelial sites. MTs are intracellular proteins with occasional nuclear localisation, and although there are persistent reports of extracellular detection of MT, the prevailing dogma is fixed that their physiological role is within cells.

MTs are metal binding proteins (61-68 amino acids), which normally bind seven zinc ions, although zinc/copper mixtures have been reported. Some isoforms are rapidly induced in response to increases in zinc or copper levels, and also by a large number of hormones and cytokines, including glucocorticoids, interleukin 1 and 6, interferons and so on. Their exact physiological role is unclear. Early suggestions that they act to prevent accumulation of toxic levels of heavy metals are no longer favoured, and if their role is indeed in metal metabolism, it is more likely that they are involved in the intracellular homeostasis of zinc. However, MTs are efficient scavengers of free radicals and are able to protect DNA and other molecules from oxidation, suggesting that their function may be protective. MTs may be considered intracellular stress proteins which respond to a wide variety of stimuli.

It is relevant that MT-I/II knockout animals, and those which overexpress MT-I and MT-II are phenotypically normal, except for sensitivity and resistance, respectively, to some chemical and physical stresses.

Deficiency of MT-III, the "brain-specific" class of MT, has been implicated in the pathogenesis of Alzheimer's disease, although this finding has been strongly disputed. MT-III reduces neuronal survival, and the applicants have shown that, when MT-III is added to cultured neurons it reduces neurite sprouting. Exogenous MT-III appears to have an opposing effect to MT-IIA and it is expected that comparison of

their structures will reveal strategies for designing analogues of both which have specific neurotrophic properties. It has been shown that exposure of rat brain lesions to MT-III causes vacuolisation, consistent with extensive neuronal death.

Metallothionein and Heavy Metals

There is a large body of literature on the relationship between metallothionein and heavy metals, particularly cadmium. MT was originally isolated as a cadmium-binding protein, and it is known that it acts as the major intracellular sink for this toxic metal. Hence, people exposed to cadmium in the workplace or through contaminated diet will have elevated metallothionein levels, particularly in the kidney. There is no question that MT acts to protect cells against cadmium, however it is not an effective agent, nor is it likely that this is the actual physiological role for the protein: it is likely an adventitious property derived from the chemical similarity between zinc and cadmium. One consequence of this is the numerous studies of the pharmacokinetics of metallothionein bound to heavy metals, following various routes of administration. Whilst cadmiummetallothionein is (not surprisingly) toxic, it is not believed that zinc or copper-metallothioneins will have significant metal-based toxicity at the concentrations employed in the studies below.

The applicants have examined the action of metallothionein proteins; including MT-IIA, a major human metallothionein of the MT-I/II class. The studies found that administration of metallothionein to cultured rat neurons increases neuronal survival and enhances the rate of axonal extension. Furthermore, in lesioned rat brains, metallothionein enhances regenerative axonal extension into the lesions and replacement of damaged tissue. Accordingly, the use of metallothionein as an active ingredient in neuronal therapy provides a novel method of stimulating neuronal growth and neuronal survival, a novel class of therapeutic agents and a novel method of treatment for a range of neuronally based disease states.

Moreover, metallothionein offers several practical advantages as a therapeutic agent.

1. It is a naturally occurring, non-toxic protein

2. It appears possible that intraperitoneally administered metallothionein can enter the CNS compartment, following physical trauma to the brain or spinal cord or breakdown of the blood-brain barrier due to other causes.

3. Metallothionein is not post-translationally modified and hence can be easily produced in bacterial or other expression systems

4. Metallothionein is a small peptide (61 amino acids for MT-IIA, 68 amino acids for human MT-III) and it is very likely that a novel analogue which is amenable to chemical synthesis can be designed.

Statement of the Invention

In one aspect the invention provides a method of stimulating neuronal growth comprising exposing a target neuron to metallothionein.

The target neuron is preferably placed in direct contact with a metallothionein solution.

The target neuron may have suffered physical trauma including lesion or other forms of neurodegeneration.

The metallothionein may be selected from any one or a combination of known metallothionein classes including MT-I, MT-II, MT-III and MT-IV and the associated isoforms.

Most preferably the metallothionein is selected from MT-II including human MT-IIA.

The metallothionein may be a synthetic analogue which combines structural or physical features of any or all known metallothionein isoforms.

The metallothionein may be provided in solution at a concentration of up to about 5μg/ml in a neurologically acceptable carrier.

Administration of the metallothionein solution may include MT-IIA as the sole active ingredient. Alternatively, any one or a combination of the metallothionein classes and isoforms as detailed above may be used. Where combinations of metallothionein are used the different classes and isoforms may be combined in a single dose. Alternatively, the different classes may be administered sequentially.

The administration regime may include initial administration of a solution of MT-IIA followed by a subsequent administration of a solution of MT-III. The administration regime may be limited to MT-IIA alone as the active ingredient.

The method of the invention may be applied to a range of compromised neuronal states including diseased states and injuries.

In another aspect the invention provides a method of treatment of any one or a combination of Alzheimers, Parkinsons, Motor Neuron Diseases, head injury, comprising the administration to a patient of a therapeutic including metallothionein as previously described as an active ingredient wherein said therapeutic is applied or administered so as to directly interact with the site of neuronal compromise.

In another aspect the invention provides a therapeutic composition comprising metallothionein in any one or a combination of isoforms, or as a synthetic metallothionein comprising features of one or more isoforms, as an active ingredient in a pharmaceutically acceptable carrier wherein said carrier is adapted for topical administration to an area of neuronal compromise.

The composition may be adapted for direct topical application to exposed neurons or for administration to non-exposed neurons by indirect routes including intravenous or intraperitoneal administration, which result in accumulation of metallothionein in the compromised region of the brain or other part of the central nervous system.

Detailed Description of the Invention

Figure 1 shows some effects of human MT-IIA on neuronal survival and neurite elongation of cultured rat cortical neurons.

Figure 2 shows by immunocytochemistry the effect of human MT-IIA on reactive neurite sprouting following axonal injury.

Figure 3 shows by immunocytochemistry the effects of human MT-IIA on reactive neurite sprouting following axonal injury.

Figures 4 and 5 show the effect of human MT-IIA on lesions in the rat neocortex formed by physical injury.

Figure 6 shows the effect of human MT-III and MT-IIA on neurite formation and initial neurite outgrowth of cultured rat cortical neurons.

Figure 7 shows the effect of human MT-III and MT-IIA on the extent and rate of neurite elongation of cultured rat cortical neurons.

Figure 8 shows the effect of human MT-III and MT-IIA on the distribution of neurite length of cultured rat cortical neurons.

Figure 9A shows immunocytochemistry the effect of human MT-III and MT-IIA on reactive neurite sprouting.

Figure 9B shows by immunocytochemistry the effect of human MT-III and MT-IIA on reactive axonal growth.

Figure 10 shows the quantitative effect of human MT-III and MT-IIA on reactive neurite sprouting and growth.

Figure 11 shows by immunohistochemistry the effect of human MT-IIA on axonal sprouting into a lesion site following physical injury in the rat neocortex.

Figure 12 shows by immunohistochemistry the effect of human MT-IIA on neuronal injury tract repair.

The action of MT-IIA (a major human metallothionein of the MT-I/-II class) in two distinct culture models of rat cortical neurons, and in a rat *in vivo* model of cortical damage was examined. In culture, it was found that administration of MT-IIA increases neuronal survival, and enhances the rate of axonal extension. In lesioned rat brains, it was found that MT-IIA enhances regenerative axonal growth into the lesion, and replacement of damaged tissue.

Metallothionein Action on Cultured Rat Cortical Neurons:

Culture Model 1: Rat cortical neurons (E18) were plated at low density in neurobasal medium + B27 supplement, including 150 μg/ml of a rat brain extract. Recombinant MT-IIA was produced (the major human metallothionein I/II isoform) in *E. coli* cultures and reconstituted as a zincthionein (7 moles zinc/mole protein).

Culture Model 2: Rat cortical neurons (E18) were plated at a higher density in Neurobasal medium + B27 supplement (but without brain extract) and cultured *in vitro* for 21 days, allowing the formation of neuronal clusters connected by fasciculated axonal bundles.

Rat Cortical Injury Model: Focal injuries were performed to the adult rat neocortex by insertion of a 25gauge beveled needle into the Par 1 region of the rat somatosensory cortex to a depth of 1.5mm into the brain.

The invention will now be described with reference to a selection of embodiments and examples and Figures 1 to 12.

Example 1

Referring in turn to Figures 1 to 7 a series of experiments were conducted using the composition made up of 0.1 to 5μg/ml of MT-IIA in a pharmaceutically and neurologically acceptable carrier. Such a composition when applied topically to a range of *in vivo* and *in vitro* neuronal situations clearly demonstrates that MT-IIA functions as an active ingredient in enhancing neurite elongation. MT-IIA also dramatically increases the extension of processes between clusters following lesions formed by microscapel and ultimately demonstrates the ability of MT-IIA to have a dramatic effect on increasing the rate of recovery from physical injuries. Referring to Figure 1, the bar graph in Figure 1A demonstrates that human MT-IIA promotes neuronal survival in the presence of adult rat brain extract (150μg/ml) after three days.

($P < 0.01$, ANOVA). Accordingly Zn-MT is not detrimental to the survival of cultured neurons.

Referring now to Figure 6E and 6F, under the same conditions human MT-IIA does not increase the initiation of new neurite sprouting over three days, expressed as either the percentage of neurite bearing neurons as shown in Figure 6E or the number of neurites per neurone as shown in Figure 6F. ($P > 0.01$ ANOVA). This observation has important clinical ramifications as inappropriate sprouting of neurons has been associated with premature neuronal death.

Referring now to Figure 7B the bar graph shows MT-IIA demonstrating dose dependent promotion of neurite elongation during this period. ($P < 0.01$, ANOVA).

From the above experiments it has been demonstrated that MT-IIA is capable of enhancing neurite elongation of cultured rat cortical neurons without increasing the rate of undesirable neurite sprouting.

Referring now to Figure 2 a culture of rat cortical neurons was maintained for twenty-one days in order to allow formation of clusters which are interconnected by fasciculated bundles of axons. The axons were cut with a microscapel and a composition as previously described, including recombinant MT-IIA, was added. The immunocytochemical results shown in Figures 2A to 2D show that twelve hours after cutting the neuronal bundles with a microscapel there is a marked retraction by transected neurites from the lesion site (which is indicated with a broken line) of up to 100μm. Whilst in the absence of MT-IIA (fig 2A), there are very few neurite extensions, as assessed by NF-M immunoreactive processes (red) extending into the area of retraction (indicated by arrows) in untreated neurons, there are many in the MT-IIA treated neurons (fig 2C). Tau and βIII-tubulin immunocytochemical analysis also indicates very few processes extending into the area of retraction (indicated by arrows) in the absence of MT-IIA (fig 2B). However, after twelve hours of incubation with MT-IIA at a concentration of 1μg/ml, these processes are significantly longer and have extended into the lesion site (fig 2D).

Referring now to Figure 3 the experiments detailed in Figure 2 were repeated with a longer exposure period of eighteen hours to recombinant MT-IIA. Eighteen hours after cutting the axonal bundles, the tissue was assessed by immunocytochemical markers and analysis of neurite extension into the lesion. In untreated samples, shown in Figure 3A, there is minimal neurite growth into lesion site. However, as shown in Figure 3C when a sample is treated with MT-IIA at a concentration of 1 μg/ml, the processes have completely traversed the lesion site with the immunoreactive processes shown in red extending from the neurite stumps indicated by arrows and have grown

towards the central lesion site indicated by the broken line. From this experimental work it has been demonstrated that MT-IIA promotes growth of NF-M immunoreactive processes which are indicated by the arrows across the central lesion site. Tau and βIII-tubulin immunocytochemical analysis also indicates that a number of processes extend into the area of retraction indicated by arrows in untreated neurons. However, these processes do not cross the transection site shown in Figure 3B. MT-IIA treated samples shown in Figure 3D have been sufficiently promoted such that the growth of processes occurs and extends beyond the transection site indicated by arrows to the opposite stump of the transected neurite bundle.

Accordingly, these experiments clearly show that MT-IIA dramatically increases the extension of processes, including axons between clusters. This occurs following lesion by microscapel and after eighteen hours of exposure to MT-IIA the axonal bundles have bridged the region between the clusters. This effect of MT-IIA is a result of a direct topical interaction between the protein, the neurons and the culture medium.

Referring now to Figures 4, 5, 11 and 12 the action of MT-IIA on a rat model of cortical injury was investigated. The rat model of physical damage to the cortex has been previously developed by the inventors and extensively characterised in terms of neuronal damage, orthology, pathology and subsequent recovery.

Figures 4, 5, 11 and 12 show the extent of the physical injury, and microglial invasion of the cavity. MT-IIA administration reduced microglial infiltration and promoted formation of a tissue bridge across the lesion, from the pial surface down. MT-IIA also promoted axonal extension into the lesion site. Very few axonal extensions were seen in the rats treated with vehicle alone (control rats).

Figure 4 shows the global location of needle stick injuries as indicated in Panel A. Brain sections underwent immunohistochemistry against SMI-312 (green) and ferritin (red) 4 days post injury. Needle stick injury resulted in a large injury tract, and microglial migration into and surrounding the injury site (B). MT-IIA treatment reduced microglial infiltration, and promoted the formation of a tissue bridge enclosing the lesion site from the pial surface down, forming a teardrop like invagination (C). Microglia at the pial surface were small and round, in contrast to the large, amoeboid microglia observed in deeper cortical layers (D, E respectively). MT-IIA promoted regenerative axonal growth into the lesion site at both the pial layer (D) and deeper cortical layers (E). In contrast, very few axonal extensions were visualised in control rats, at the pial level (F) or deeper cortical layers (G). Arrowheads indicate the injury tract.

Figure 5 shows immunohistochemical staining against SMI-312 (green) and ferritin (red) 4 days post injury. SMI-312 immunoreactive axonal extensions were often found in close association with small round microglia at the pial surface (A, B). Contrastingly, axonal extensions in deeper cortical layers were often not associated with larger, amoeboid microglia (C, D). Regenerating axons often exhibited a wavy morphology, as if they were constantly changing direction. Occasionally, pyramidal (indicated by arrow, C) and bulb-like (indicated by arrow, D) accumulations were observed along axonal sprouts.

The above experiments clearly indicated that the administration of recombinant MT-IIA dramatically increases the rate of recovery from physical injuries. In combination with the tissue culture experiments, this work indicates that the administration of MT-IIA following central nervous system injury acts directly on neurons to increase the rate of axonal extension into the lesion.

Example 2

Turning now to the effect of human MT-III as contrasted to MT-IIA, a series of experiments were conducted as detailed in Figures 6 to 12. Referring firstly to Figure 6, the effect of MT-III on neurite formation is shown in Figure 6A in the presence of adult rat brain extract at 150μg/ml after three days. Figure 6B shows neurite bearing neurons indicated by the arrows. The percentage of neurite bearing neurons is shown in Figure 6C and the number of neurites per neurite-bearing neuron is shown in Figure 6D. From the above it can be seen that MT-III significantly inhibits neurite outgrowth in both instances at the concentrations tested ($p < 0.01$, ANOVA). Referring now to Figure 6E human MT-IIA had no effect on initial neurite outgrowth over three days. As assessed by both the percentage of neurite bearing neurons or as detailed in Figure 6F, the number of neurites per neurite-bearing neurons. Referring now to Figure 7, it has been shown that human MT-III prolongs the process of neurite retraction from 0-2 and 2-4 hours after plating as can be seen with reference to Figure 7A. Following this, the rate of neurite elongation is reduced. Referring now to Figure 7B, in contrast to this, application with human MT-IIA significantly increases the rate of neurite elongation.

The distribution of neurite lengths three days after MT-III treatment is shown in Figure 8A, where it is clearly indicated that while MT-III significantly inhibits neurite growth, a small percentage of neurites were unaffected and grew to lengths comparable to vehicle treated neurites. In contrast to this the distribution of neurite lengths

following MT-IIA treatment indicated that a number of neurites grew to lengths greater than that of vehicle treated neurites as can be seen with reference to Figure 8B.

Fluorescent double immunocytochemical labelling of cytoskeletal changes both tau shown in red and βIII-tubulin shown in green twelve hours after axonal transection are shown in Figures 9A Panel A and 9A Panel B. In contrast to this, treatment with MT-III reduced regenerative neurite sprouting compared to vehicle treated samples as shown in Figures 9A Panel C and 9A Panel D .

The transection site is indicated by a broken line and there is a large area of retraction away from this line. Sprouting neurites are indicated by arrows. The MT-IIA treatment increased both the number and length of reactive sprouts following injury and this is detailed in Figure 9A Panel E which details the vehicle example and 9A Panel F which indicates the MT-IIA example at 1μg/ml (check, please).

Referring now to the eighteen hours post axonal transection. This is shown by flourescent double immunocytochemcial labelling of cytoskeletal changes, both tau shown in red and βIII-tubulin shown in green. The MT-IIA can be seen to have promoted reactive axonal growth across the entire transection site shown in Figure 9B Panel A. In contrast such axonal growth is not observed following treatment with either vehicle as shown in Figure 9B Panel B or MT-III shown in 9B Panel C.

Treatment of samples with human MT-III significantly inhibited both the number and length of reactive sprouts at a twelve hour interval after axonal transection in culture. However, treatment with human MT-IIA significantly increased the mean neurite length of reactive sprouts at 12 hours after transection . These findings are detailed in Figures 10A and 10B.

Example 3

Further experimental work was done to test the effect of MT-IIA and these results are shown in Figures 11 and 12 under immunohistochemical studies for SMI-312 (green axonal marker) and ferritin (red microglial marker). Referring firstly to Figure 11, the results at four days post injury are shown where the needle stick injury resulted in a large injury tract and microglial migration into and surrounding the injury site shown in Figure 11A. MT-IIA treatment promoted the formation of a tissue bridge enclosing the lesion site from the pial surface down so as to form a tear drop like invagination shown in Figure 11B. MT-IIA further promoted axonal sprouting into the lesion site at both the pial layer as shown in Figure 11C and deeper cortical layers shown in Figure 11D. In contrast to this, very few axonal sprouts were visualised in

control rats as shown at the pial level in Figure 11E and deeper cortical layers as shown in Figure 11F. The arrow heads indicate the injury tract.

Figure 12 shows details of further experimental work on brain sections at 7 days post injury. In vehicle treated rats, the injury tract was smaller compared to four days post injury shown in Figure 11; although it had not completely closed over. a degree of reactive sprouting is evident in all animals at this time point as shown in Figure 12A. Reactive processes exhibited greater SMI-312 reactivity than uninjured neuronal processes in surrounding neural tissue. Reactive astrocytes also aligned along the borders of the injury tract as shown in Figure 12B. In MT-IIA treated rats, the entire injury tract had closed over, and was demarcated only by a fine line of ferritin immunoreactivity as shown in Figure 12C. Reactive astrocytes also enclose the injury tract, and were found at lower density in adjacent uninjured tissue shown in Figure 12D. In MT-IIA treated animals, numerous reactive axonal processes were observed as shown by the arrows within the injury tract at both deeper cortical levels shown in Figure 12E and pial levels shown in Figure 12F .

The above detailed examples demonstrate the clinical application of MT-IIA in promoting nerve cell survival, promoting neuronal regeneration and generally enhancing neurite elongation without causing inappropriate neuronal sprouting.

The findings and experimental results support many clinical applications of the invention as detailed below.

Disease	**Indication**	**Role of MTI/II (IIA)**
Alzheimer's disease	Promote nerve cell survival. Promote neuronal regeneration. Buffer metals implicated in development of pathological hallmarks.	It was demonstrated that MTI/II is upregulated in early stages of the disease (published)
Parkinson's disease	Promote nerve cell survival. Promote regeneration. Buffer metals implicated in toxicity.	Evidence of abnormal metal homeostasis in the brain as well as neurodegeneration.
Motor neuron disease	Promote nerve cell survival. Promote neuronal regeneration. Buffer metals implicated in toxicity. Reduce oxidative stress implicated in neuronal degeneration.	Evidence of abnormal metal homeostasis in the brain and spinal cord as well as neurodegeneration.

Head injury	Promote nerve cell survival. Promote neuronal regeneration.	It was shown that MT I/II is upregulated at zone of injury. Recombinant protein promotes brain healing and axonal regeneration
Spinal cord trauma	Promote nerve cell survival. Promote neuronal regeneration.	Evidence of delayed neurodegeneration and spinal cavitation following injury. The recombinant protein is potentially capable of promoting neural healing and regeneration.
Glaucoma	Promote nerve cell survival. Promote neuronal regeneration.	Axonal damage followed by neurodegeneration underlies the disease. MTI/II may potentially promote survival of nerve cells and/or appropriate regeneration.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS

1. A method of stimulating neuronal growth or repair comprising exposing a target neuron or neuronal area to a solution of the metallothionein isoform MT-IIA.

2. A method according to claim 1 wherein said contact is by direct interaction of the target neuron or neuronal site with said solution.

3. A method according to claim 1 or 2 wherein said MT-IIA is naturally occurring human MT-IIA.

4. A method according to claim 1 or 2 wherein said MT-IIA is produced by chemical synthesis or by production in genetically manipulated cells or organisms.

5. A method according to claim 4 wherein said MT-IIA is recombinant human MT-IIA.

6. A method according to any one of claims 1 to 5 wherein said solution has a concentration of up to about 5μg/ml metallothionein in a neurologically acceptable carrier.

7. A method according to claim 6 wherein said solution has a concentration of about 5 μg/ml metallothionein in solution.

8. A method according to any one of claims 1 to 5 further including exposing said neuron or neuronal area to any one or a combination of metallothionein isoforms selected from MT-I, MT-II, MT-III and MT-IV.

9. A method according to claim 8 wherein said target neuron or neuronal area is exposed simultaneously to a combination of MT-IIA and any one or a combination of metallothionein isoforms selected from MT-I, MT-II, MT-III and MT-IV.

10. A method according to claim 8 wherein said target neuron or neuronal area is exposed sequentially to a combination of MT-IIA followed by any one or a combination of metallothionein isoforms selected from MT-I, MT-II, MT-III and MT-II.

11. A method according to claim 8 wherein said target neuron or neuronal area is exposed sequentially to a combination of any one of metallothionein isoforms selected from MT-I, MT-II, MT-IIA, MT-III and MT-IV.

12. A method according to any one of claims 1 to 11 wherein said neuron or neuronal area is located in the brain.

13. A method according to any one of claims 1 to 12 wherein said solution is administered to said neuron or neuronal area by any one or a combination of direct injection, intraperitoneal injection, oral administration or via genetically modified cells including stem cells.

14. A method of treatment of Alzheimer's Disease comprising administration to a patient in need of treatment a therapeutic composition including metallothionein in accordance with the method of any one of claims 1 to 13.

15. A method of treatment of Parkinson's Disease comprising administration to a patient in need of treatment a therapeutic composition including metallothionein in accordance with the method of any one of claims 1 to 13.

16. A method of treatment of motor neuron disease comprising administration to a patient in need of treatment a therapeutic composition including metallothionein in accordance with the method of any one of claims 1 to 13.

17. A method of treatment of head injury comprising administration to a patient in need of treatment a therapeutic composition including metallothionein in accordance with the method of any one of claims 1 to 13.

18. A therapeutic composition adapted for topical administration to an area of neuronal compromise said composition characterised by metallothionein isoform MT-IIA as an active ingredient.

19. A composition according to claim 18 wherein said active ingredient is combined with any one or a combination of metallothionein isoforms selected from MT-I, MT-II, MT-III and MT-IV.

20. A composition according to claim 18 or 19 wherein said metallothionein is naturally occurring human MT-IIA.

21. A composition according to any one of claims 18 or 19 wherein said metallothionein is produced by chemical synthesis or by production in genetically manipulated cells or organisms.

22. A composition according to claim 21 wherein said metalliothionein is recombinant human MT-IIA.

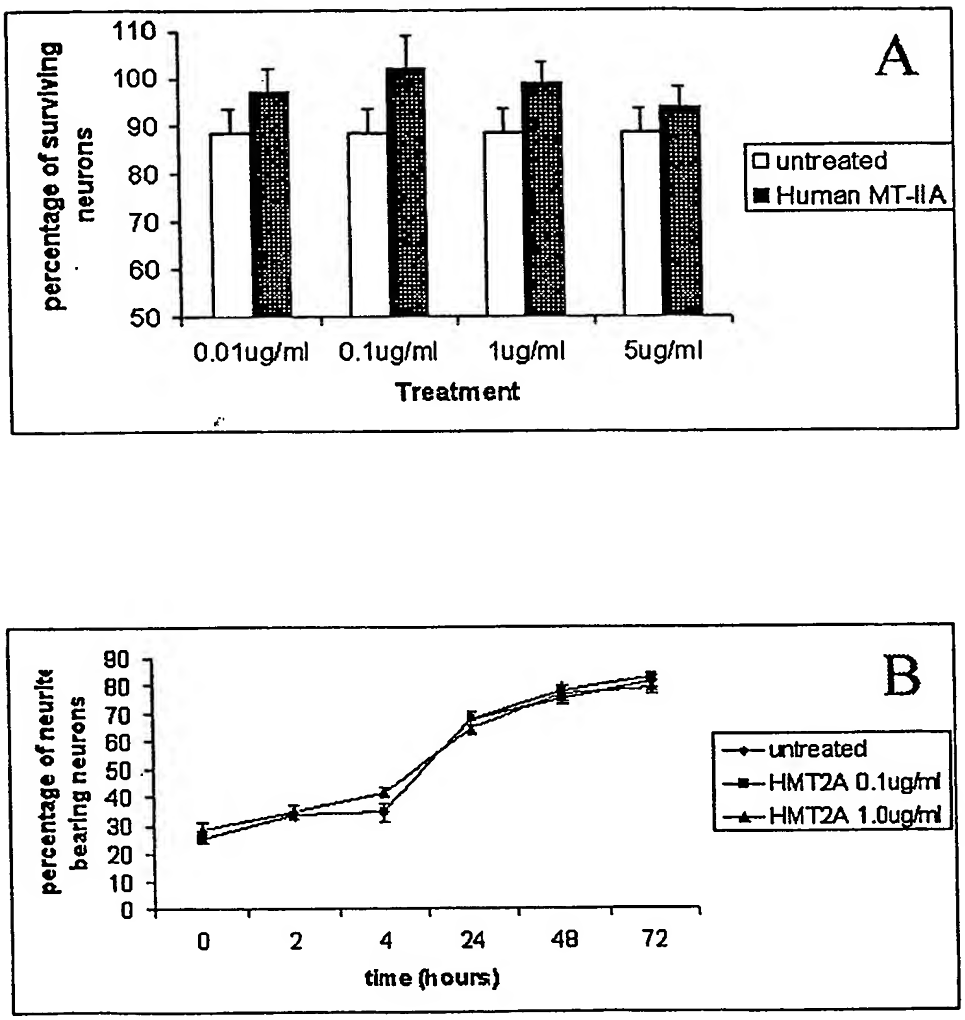
23. A composition according to any one of claims 18 to 22 further including a neurologically acceptable carrier particularly adapted for a topical administration to an area of neuronal compromise.

24. A composition according to claim 23 adapted for direct topical application.

25. A composition according to claim 23 adapted for intraperitoneal or intravenous administration to effect exposure of neurons by a non-topical route

26. A method according to any one of claims 1 to 17 substantially as hereinbefore described with reference to the examples.

27. A composition according to any one of claims 18 to 25 substantially as hereinbefore described with reference to the examples.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Treatment
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Human MT-IIA
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percentage of neurite bearing neurons
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80
70
60
50
40
30
20
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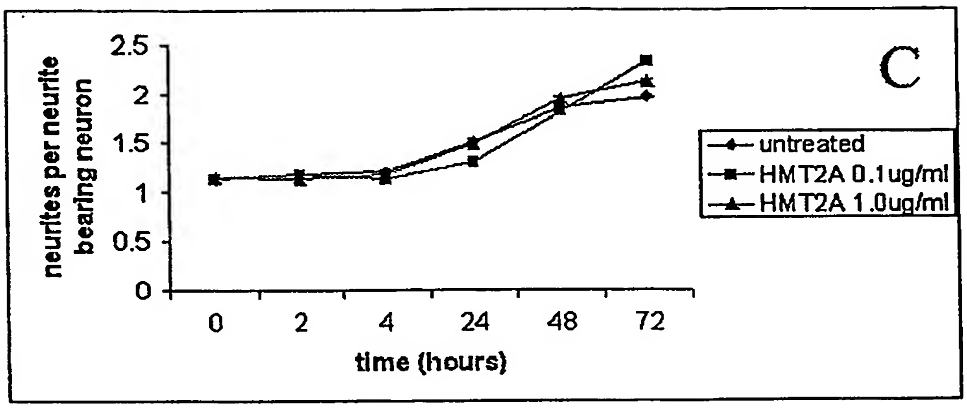
HMT2A 1.0ug/ml

Figure 1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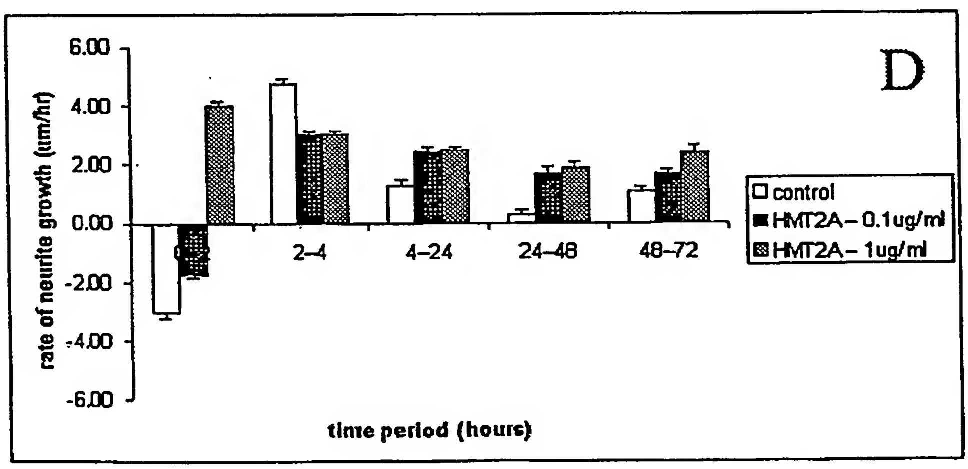
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rate of neurite growth (um/hr)
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time period (hours)
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Figure 1

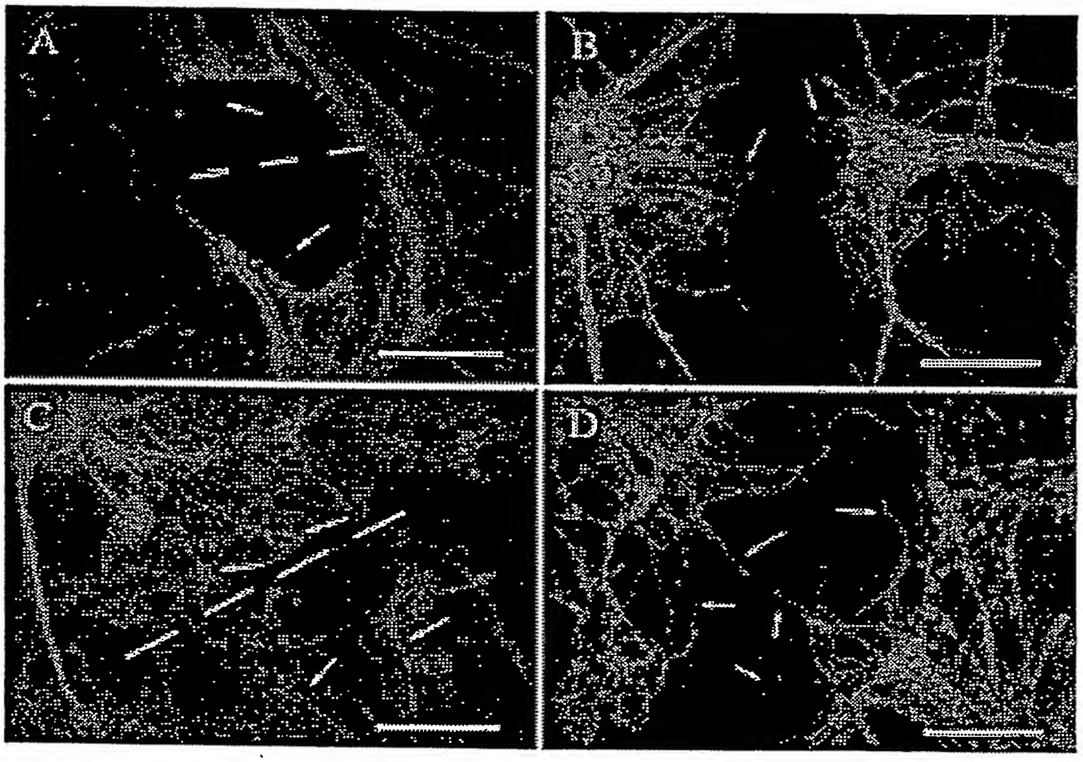


Figure 2

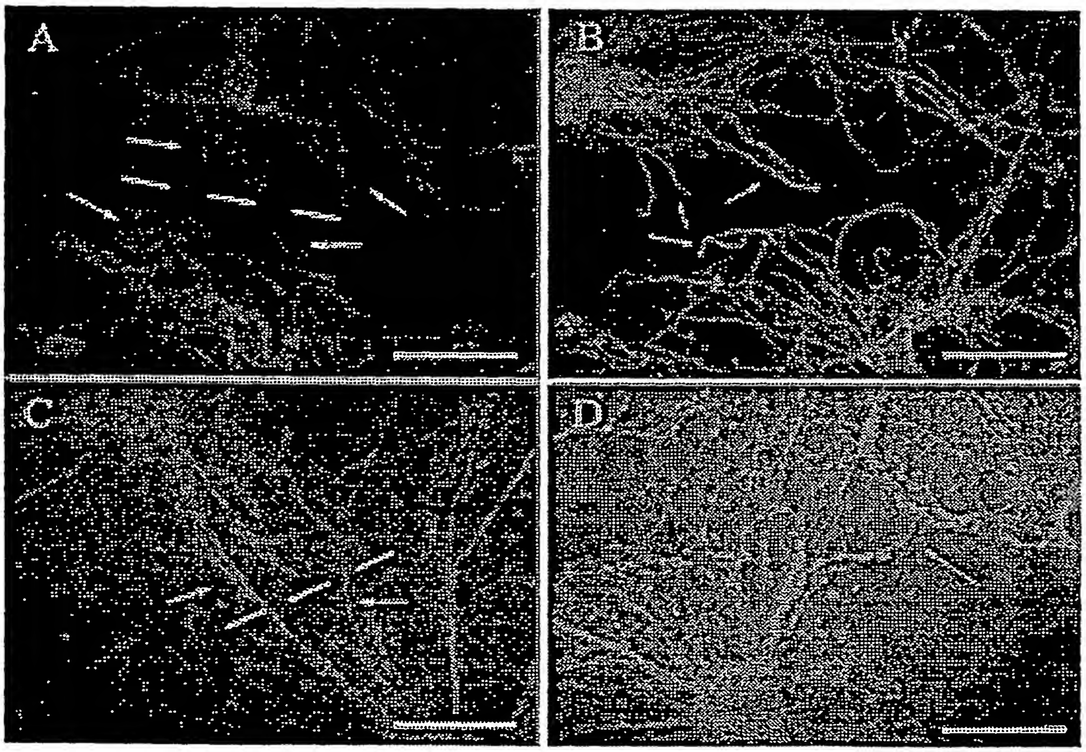


Figure 3

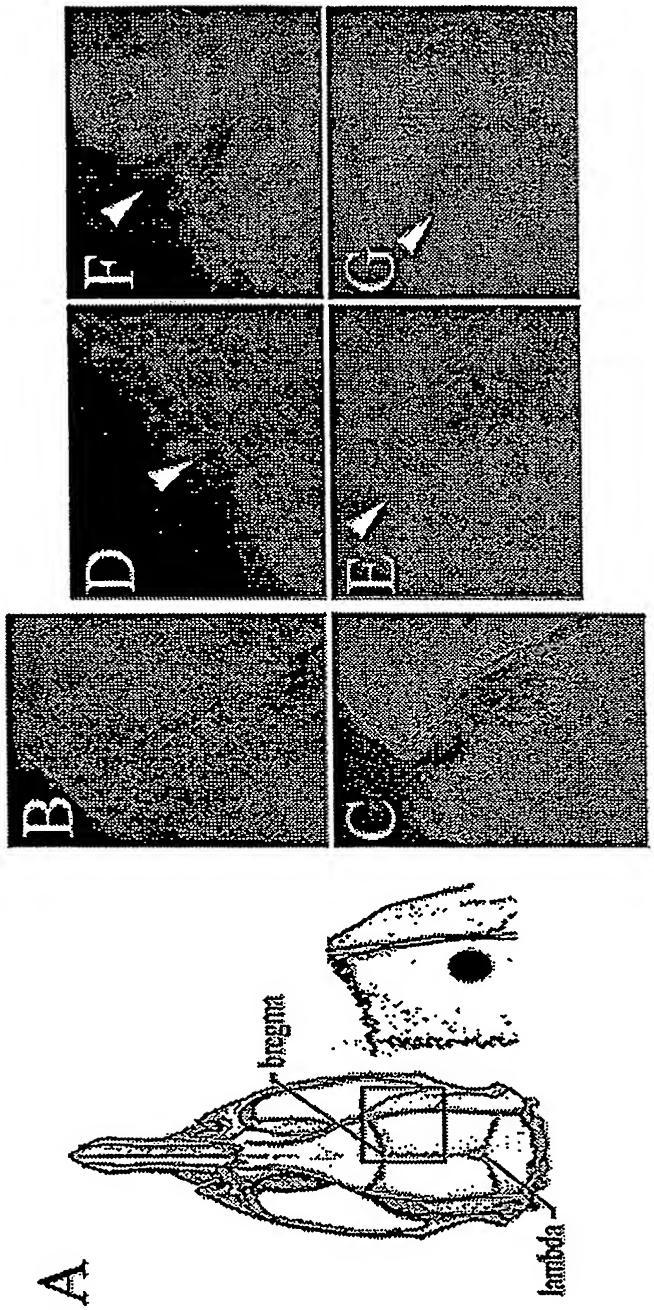


Figure 4

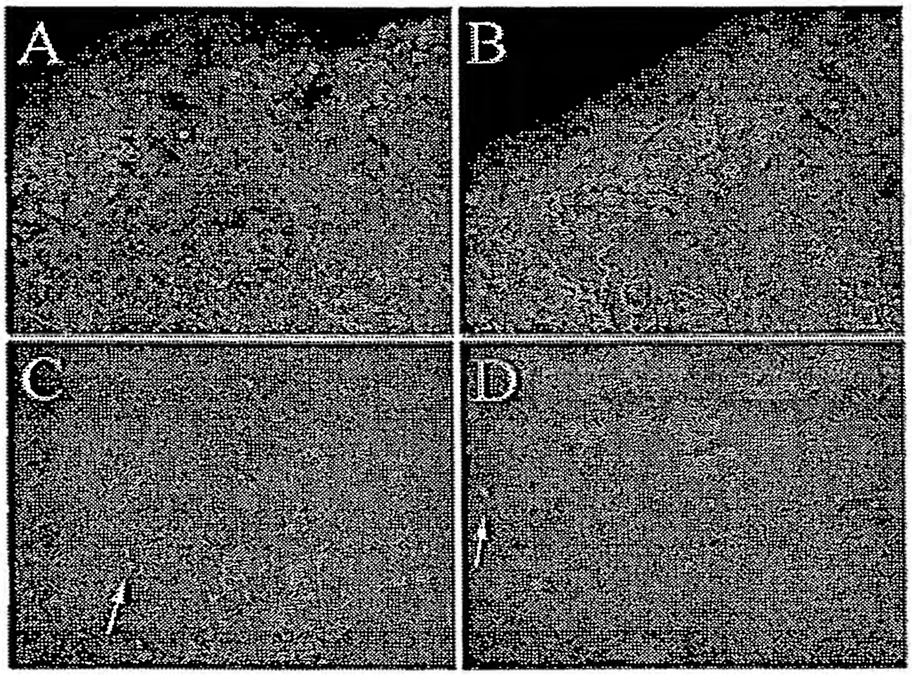


Figure 5

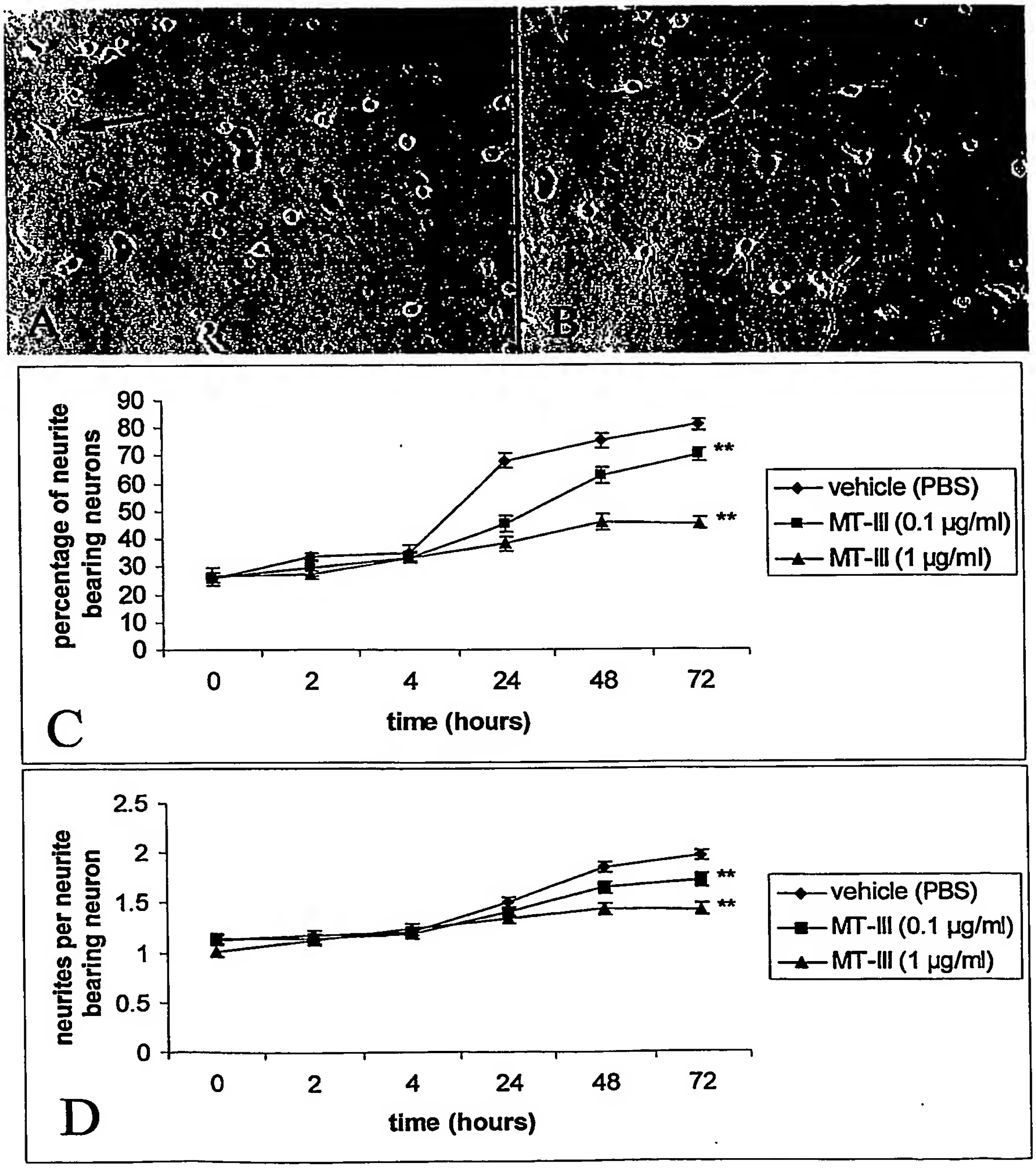

A
B
percentage of neurite bearing neurons
90
80
70
60
50
40
30
20
10
0
0
2
4
24
48
72
time (hours)
**
**
vehicle (PBS)
MT-III (0.1 µg/ml)
MT-III (1 µg/ml)
C
neurites per neurite bearing neuron
2.5
2
1.5
1
0.5
0
0
2
4
24
48
72
time (hours)
**
**
vehicle (PBS)
MT-III (0.1 µg/ml)
MT-III (1 µg/ml)
D

Figure 6

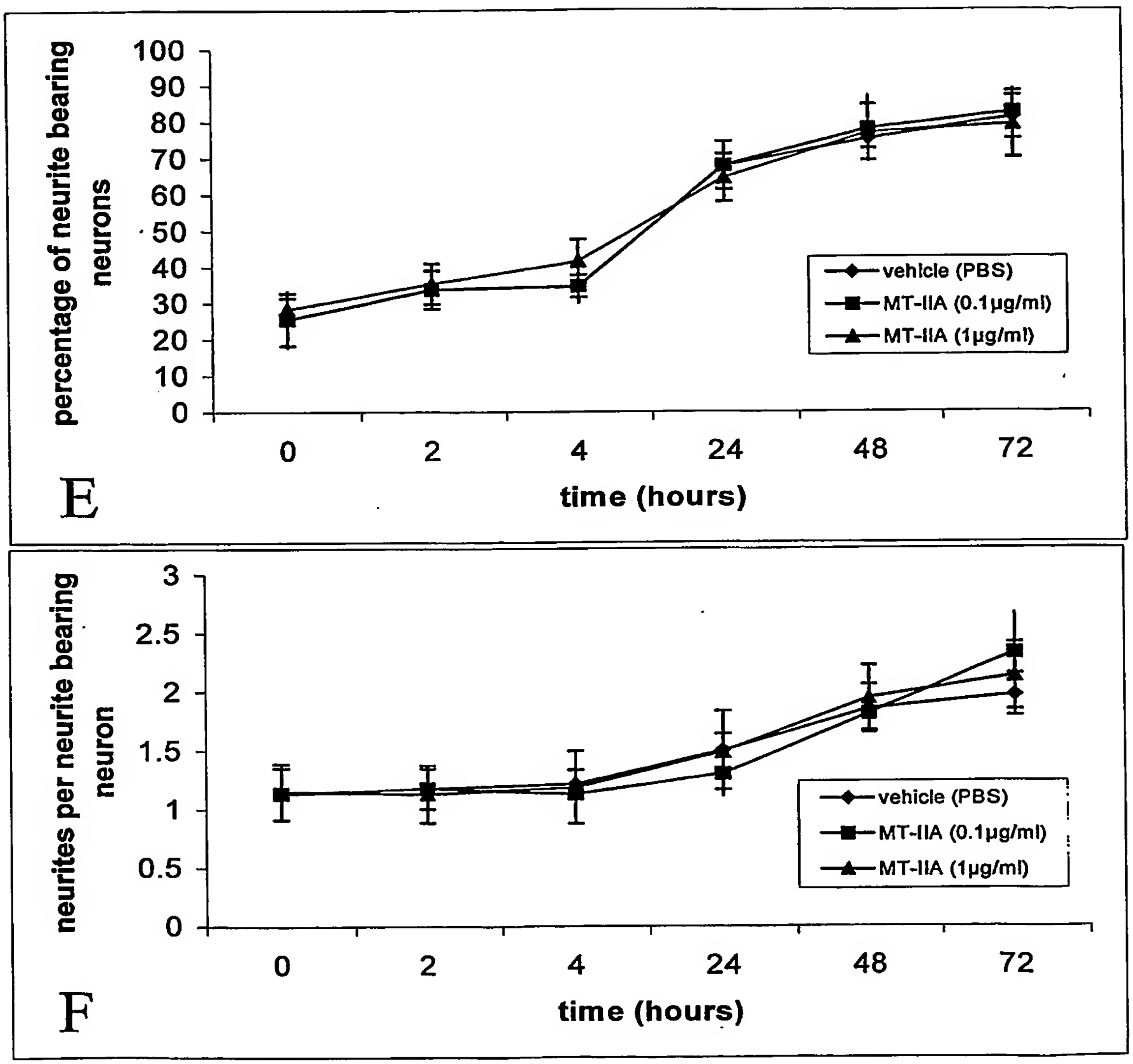
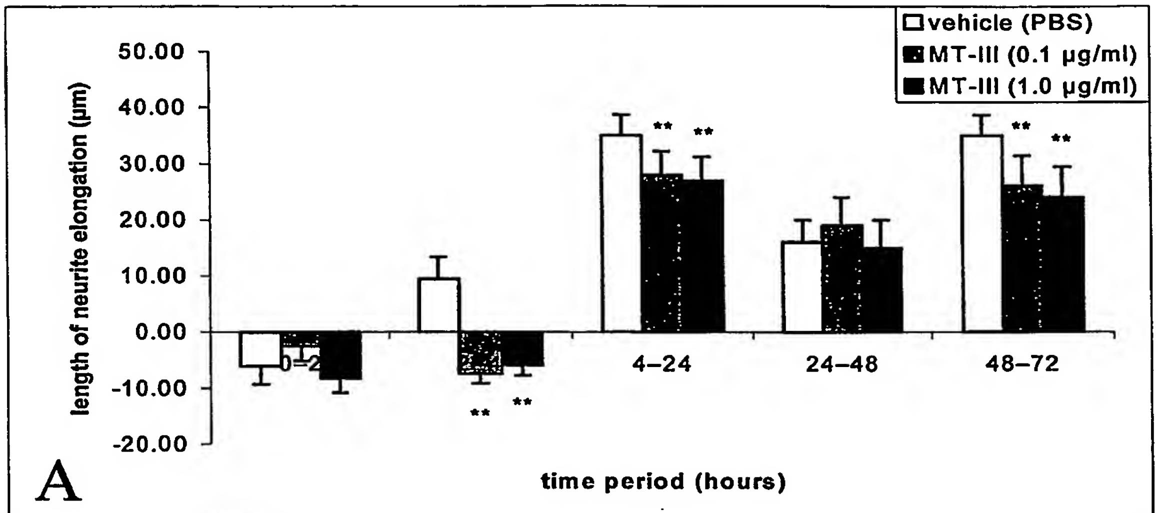

percentage of neurite bearing neurons
100
90
80
70
60
50
40
30
20
10
0
0
2
4
24
48
72
time (hours)
vehicle (PBS)
MT-IIA (0.1µg/ml)
MT-IIA (1µg/ml)
E
neurites per neurite bearing neuron
3
2.5
2
1.5
1
0.5
0
0
2
4
24
48
72
time (hours)
vehicle (PBS)
MT-IIA (0.1µg/ml)
MT-IIA (1µg/ml)
F

Figure 6


vehicle (PBS)
MT-III (0.1 µg/ml)
MT-III (1.0 µg/ml)
length of neurite elongation (µm)
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
-20.00
0–2
4–24
24–48
48–72
time period (hours)
A

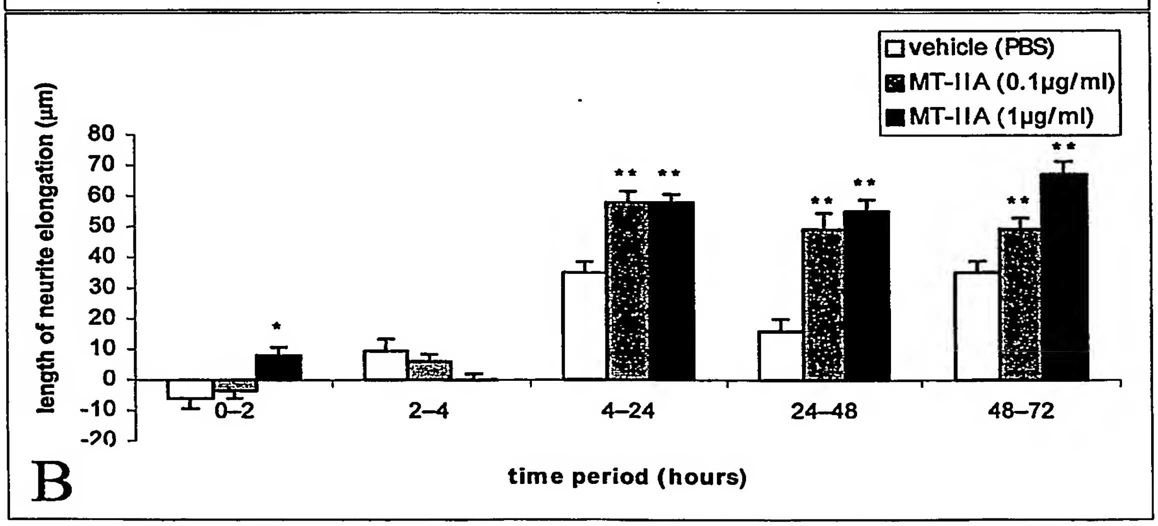
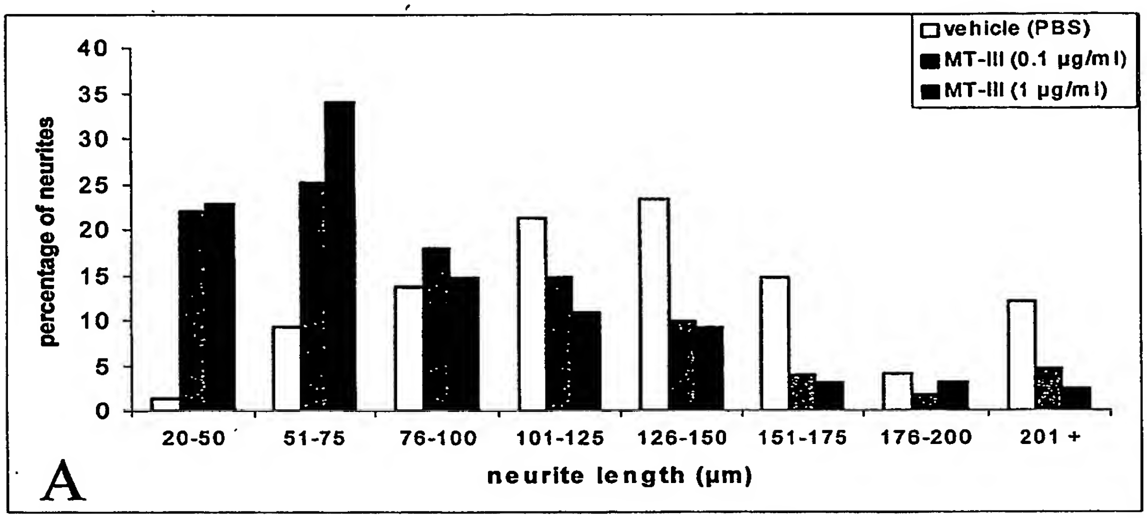

vehicle (PBS)
MT-IIA (0.1µg/ml)
MT-IIA (1µg/ml)
length of neurite elongation (µm)
80
70
60
50
40
30
20
10
0
-10
-20
0–2
2–4
4–24
24–48
48–72
time period (hours)
B

Figure 7


vehicle (PBS)
MT-III (0.1 µg/ml)
MT-III (1 µg/ml)
percentage of neurites
40
35
30
25
20
15
10
5
0
20-50
51-75
76-100
101-125
126-150
151-175
176-200
201 +
neurite length (µm)
A

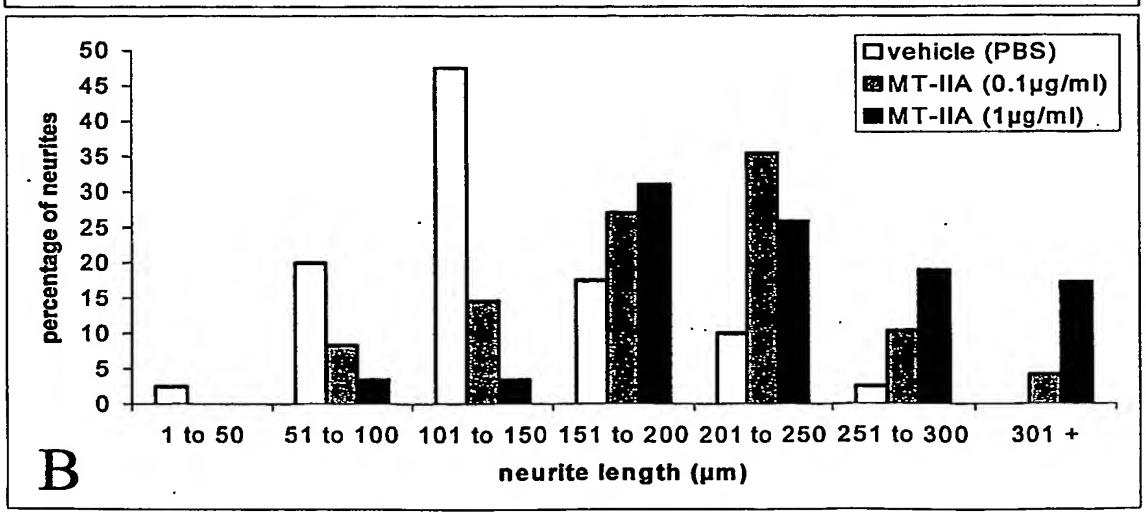

vehicle (PBS)
MT-IIA (0.1µg/ml)
MT-IIA (1µg/ml)
percentage of neurites
50
45
40
35
30
25
20
15
10
5
0
1 to 50
51 to 100
101 to 150
151 to 200
201 to 250
251 to 300
301 +
neurite length (µm)
B

Figure 8

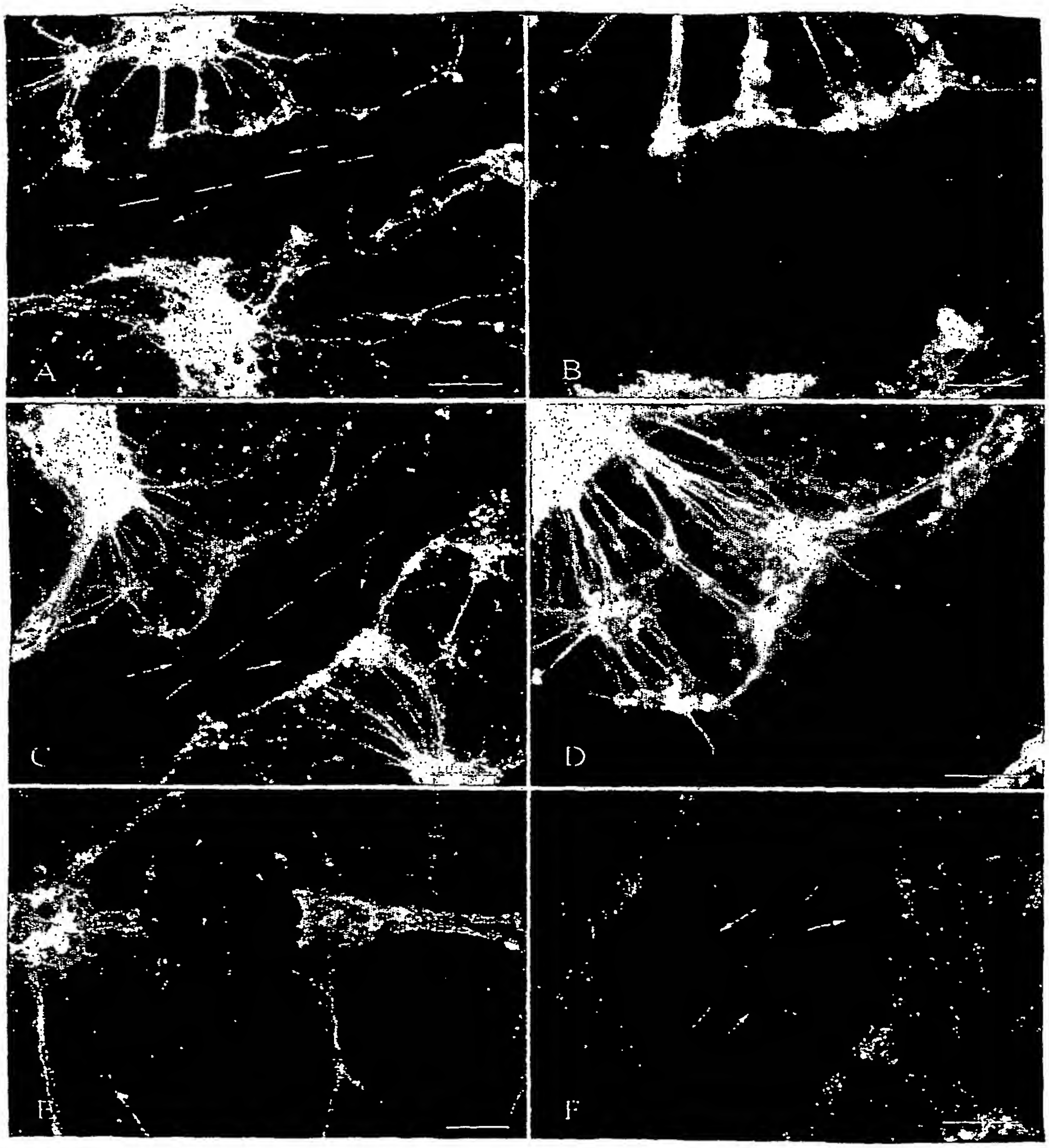
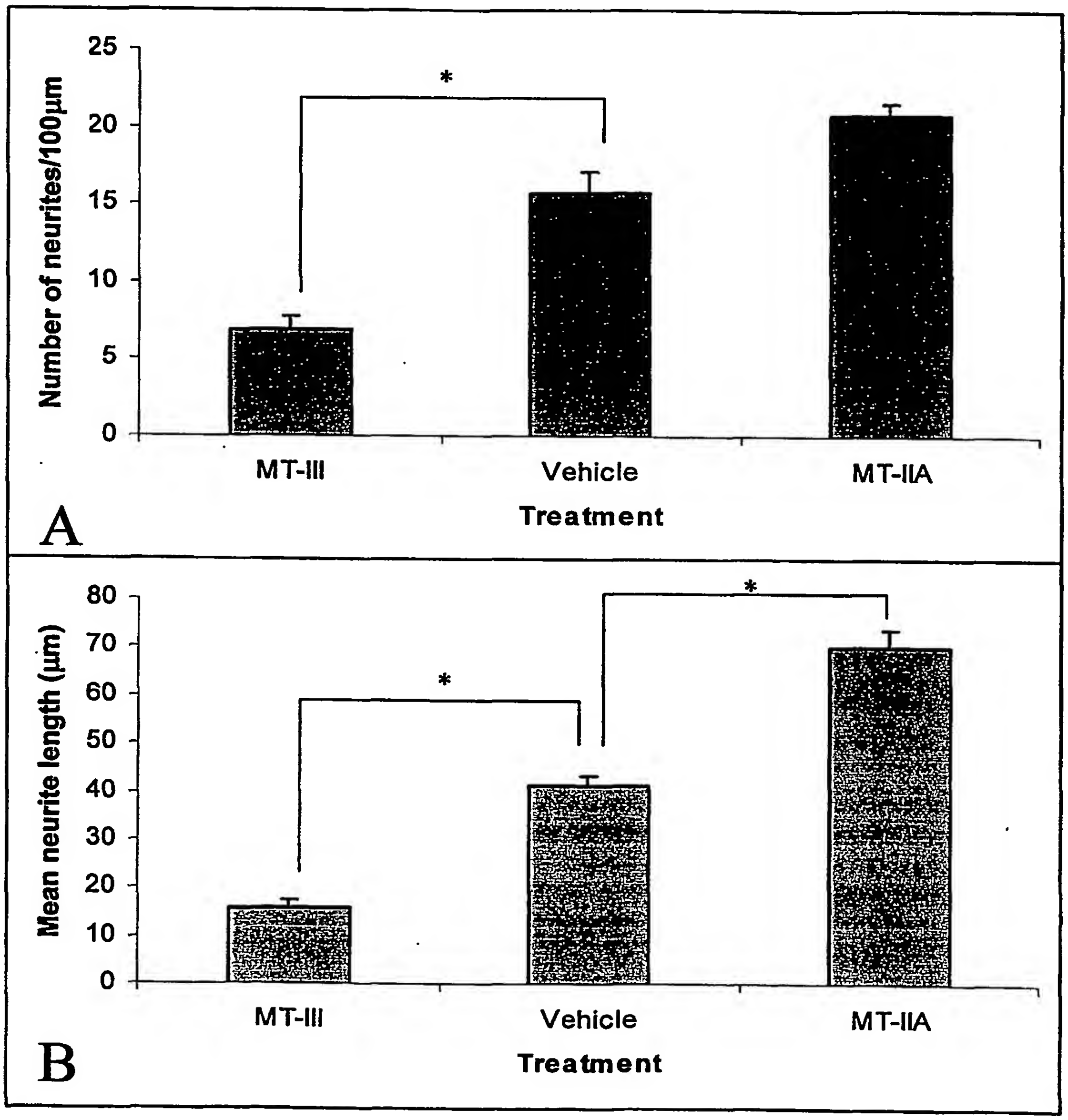


Figure 9A

Figure 9B



25
20
15
10
5
0
Number of neurites/100µm
*
MT-III
Vehicle
MT-IIA
Treatment
A
80
70
60
50
40
30
20
10
0
Mean neurite length (µm)
*
*
MT-III
Vehicle
MT-IIA
Treatment
B

Figure 10

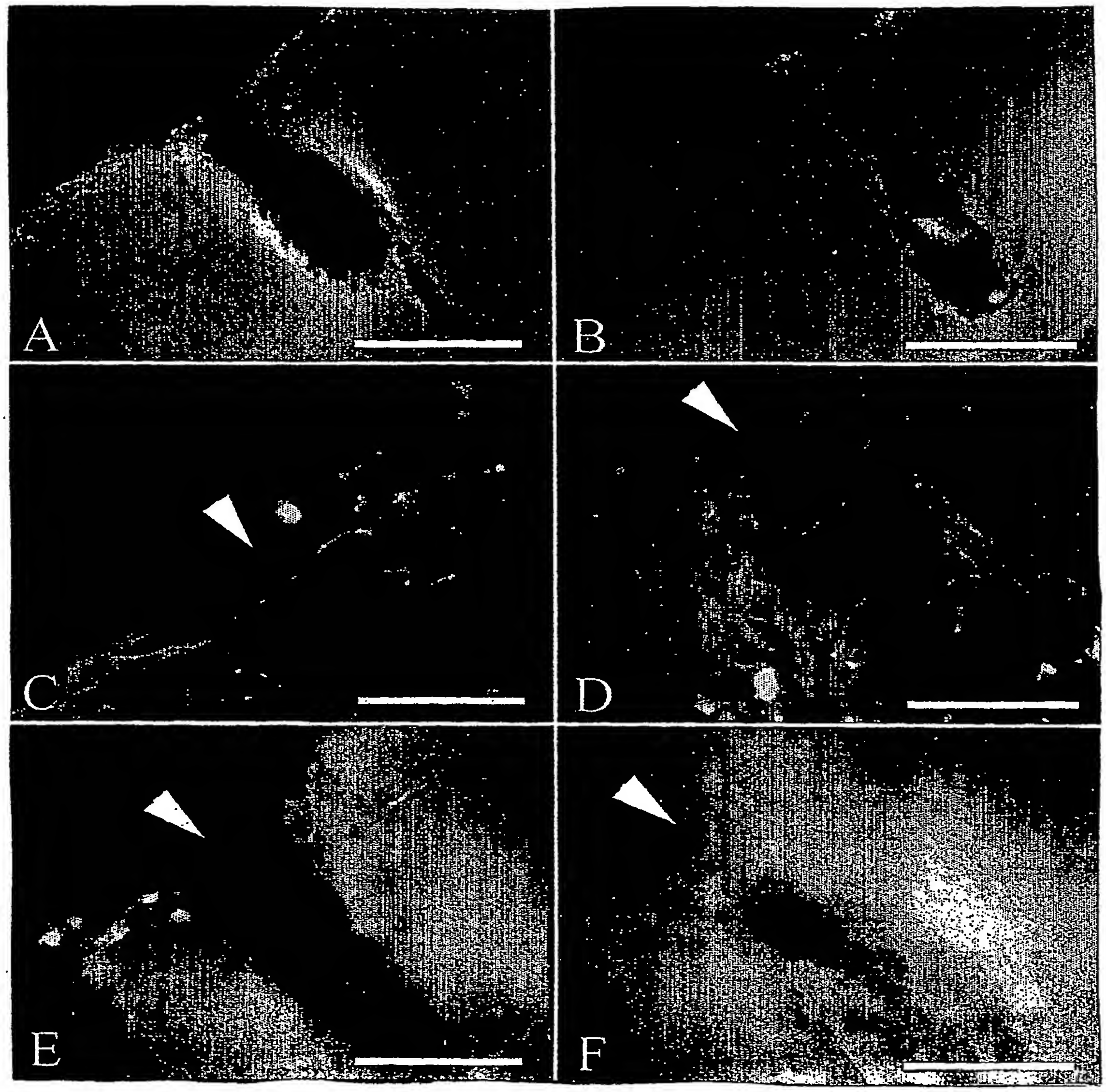


Figure 11

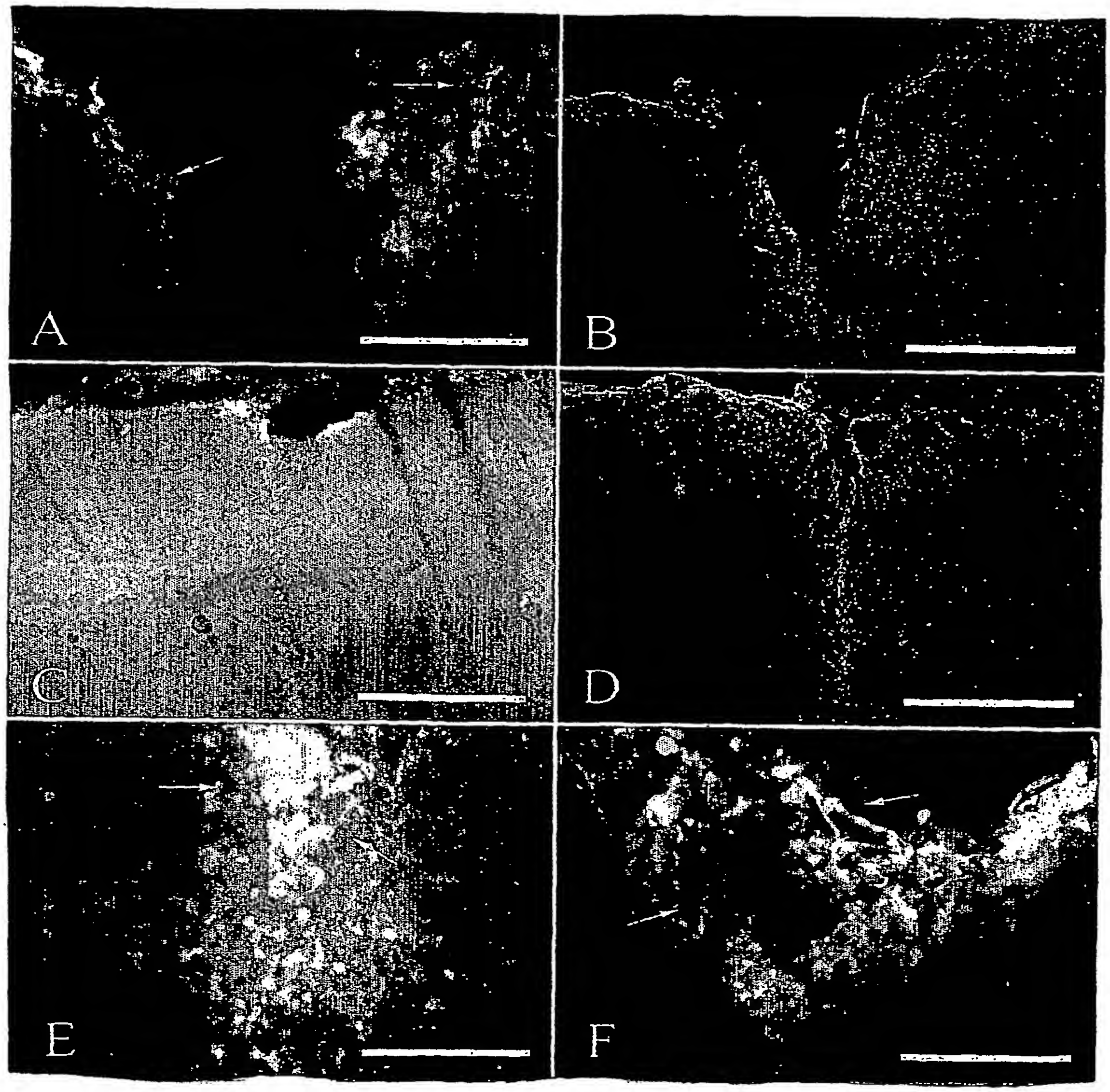


Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00735

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: A61K 038/17; A61P 25/16, 25/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DWPI, Medline: metallothionein, MT-IIA, MT IIA, MTIIA, neuron, alzheimer, parkinson

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,268,175A (BOMBARDELLI, Ezio et al.) 7 December 1993 See whole document	18-25, 27
X	US 5,431,923A (BOMBARDELLI, Ezio et al.) 11 July 1995 See whole document	18-25, 27
X	FR 2813529 (Provital, S.A.) 8 March 2002 See whole document	18-25, 27

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2003	- 6 AUG 2003
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer MICHAEL GRIEVE Telephone No : (02) 6283 2267

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00735

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 98/31795A (Incyte Pharmaceuticals, Inc.) 23 July 1998	
X	See whole document	18-25, 27
Y		1-17, 26
	WO 02/43507A (The Health Research Institute) 6 June 2002	
Y	See whole document	1-17, 26
	Richarz, A-N et al. "Speciation Analysis of Trace Elements in the Brains of Individuals with Alzheimer's Disease with Special Emphasis on Metallothioneins" Anal Bioanal Chem Vol.372(3) (2002) pages 412 to 417	
Y	See whole document	1-27
	Lui, E. et al. "Metals and the Liver in Alzheimer's Disease: An Investigation of Hepatic Zinc, Copper, Cadmium, and Metallothionein" J Am Geriatr Soc Vol.38(6) (1990) pages 633 to 639	
Y	See whole document	1-27
	Aldard, P.A. et al. "Increased Density of Metallothionein I/II-Immunopositive Cortical Glial Cells in the Early Stages of Alzheimer's Disease" Neurobiology of Disease Vol.5(5) (1998) pages 349 to 356	
Y	See whole document	1-27
	Zambenedetti, P. et al. "Metallothioneins are Highly Expressed in Astrocytes and Microcapillaries in Alzheimer's Disease" Journal of Chemical Neuroanatomy Vol.15(1) (1998) pages 21 to 26	
Y	See whole document	1-27
	Aschner, M. "The Functional Significance of Brain Metallothioneins" The FASEB Journal Vol.10(10) (1996) pages 1129 to 1136	
Y	See whole document	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU03/00735

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	5268175	CA	2089530	DE	4212134	EP	557042
		HK	1399/96	JP	6179626	SG	48724
		US	5431923				
US	5431923	CA	2089530	DE	4212134	EP	557042
		HK	1399/96	JP	6179626	SG	48724
		US	5268175				
FR	2813529	WO	200215344				
WO	200243507	AU	200236527	US	2002155170		
WO	9831795	AU	59047/98	EP	953049	US	5814480
		US	5955428				

END OF ANNEX